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Extensive mitochondrial DNA variation in somatic tissue cultures initiated from wheat immature embryos

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Summary. Wheat mitochondrial DNA has been isolated from callus cultures initiated from both immature embryos and the corresponding parental cultivar. A SalI restriction pattern study has shown that the organization of callus culture mitochondrial DNA underwent extensive change, characterized by either the disappearance or the decrease in the relative stoichiometry of several restriction bands. Hybridization of labelled mitochondrial fragments obtained from a recombinant cosmid library to Southern blots of callus and parental line restricted mitochondrial DNAs has shown that a fraction of the mitochondrial genome was lost in callus cultures. Data from a SalI + HindIII restriction map of a defined part of the wheat mitochondrial genome concerned with some of these variations strongly suggest that the observed variations correspond to the disappearance of at least one mitochondrial DNA subgenomic molecule in callus cultures.

Key words: *Triticum aestivum* – Tissue culture – Mitochondrial DNA – Genomic variability

Introduction

Since the appearance of the first reports showing the complexity of higher plant mitochondrial DNA (Levings and Pring 1976; Quétier and Vedel 1977), the extensive use of restriction endonucleases has revealed the higher plant mitochondrial genome to be very large and composed of a heterogeneous population of DNA molecules. Furthermore, comparative mtDNA restriction pattern analyses of plant cell cultures and corresponding whole plants have shown that in some cases rearrangements occur within the mitochondrial genome of in vitro cultures (McNay et al. 1984; DeBonte et al. 1984; Negruk et al. 1986). Changes in the relative stoichiometry of restriction bands seem to be the most frequent variation observed (McNay et al. 1984; DeBonte et al. 1984). However, both transposition of defined DNA fragments (Kemble and Mans 1983) and recombination after heteroplasmic protoplast fusion (Belliard et al. 1979; Nagy et al. 1983) have already been reported. Mutations or deletions within restriction sites could also lead to the appearance of novel bands concomitent with the disappearance of restriction bands as observed in whole plant mtDNA by McNay et al. 1984. As pointed out by Kemble and Shepard (1984), higher plant tissue culture may be regarded as a process which increases the molecular diversity of the mitochondrial genome. It has also been suggested that this phenomenon could reflect changes in the efficiency of expression of different genes (Spruill et al. 1980; Leaver and Gray 1982). However, the mitochondrial rearrangements following in vitro tissue culture do not occur systematically since in several cases (Dale et al. 1981; Galun et al. 1982) no change has been detected in the restriction patterns of cell culture mtDNA.

Most of the results reported so far on mtDNA variation arise from the comparison of restriction patterns. It is obvious that, when possible, the use of cloned mtDNA fragments as labelled probes together with the knowledge of their intramolecular arrangement represents a suitable tool for precisely detecting and locating rearrangements occurring within the mitochondrial genome. Data obtained in our laboratory from a SalI

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Abbreviations: *mtDNA* mitochondrial DNA; *cpDNA* chloroplast DNA; *rRNA* ribosomal RNA; *mRNA* messenger RNA; *kb* kilobase; *cv* cultivar

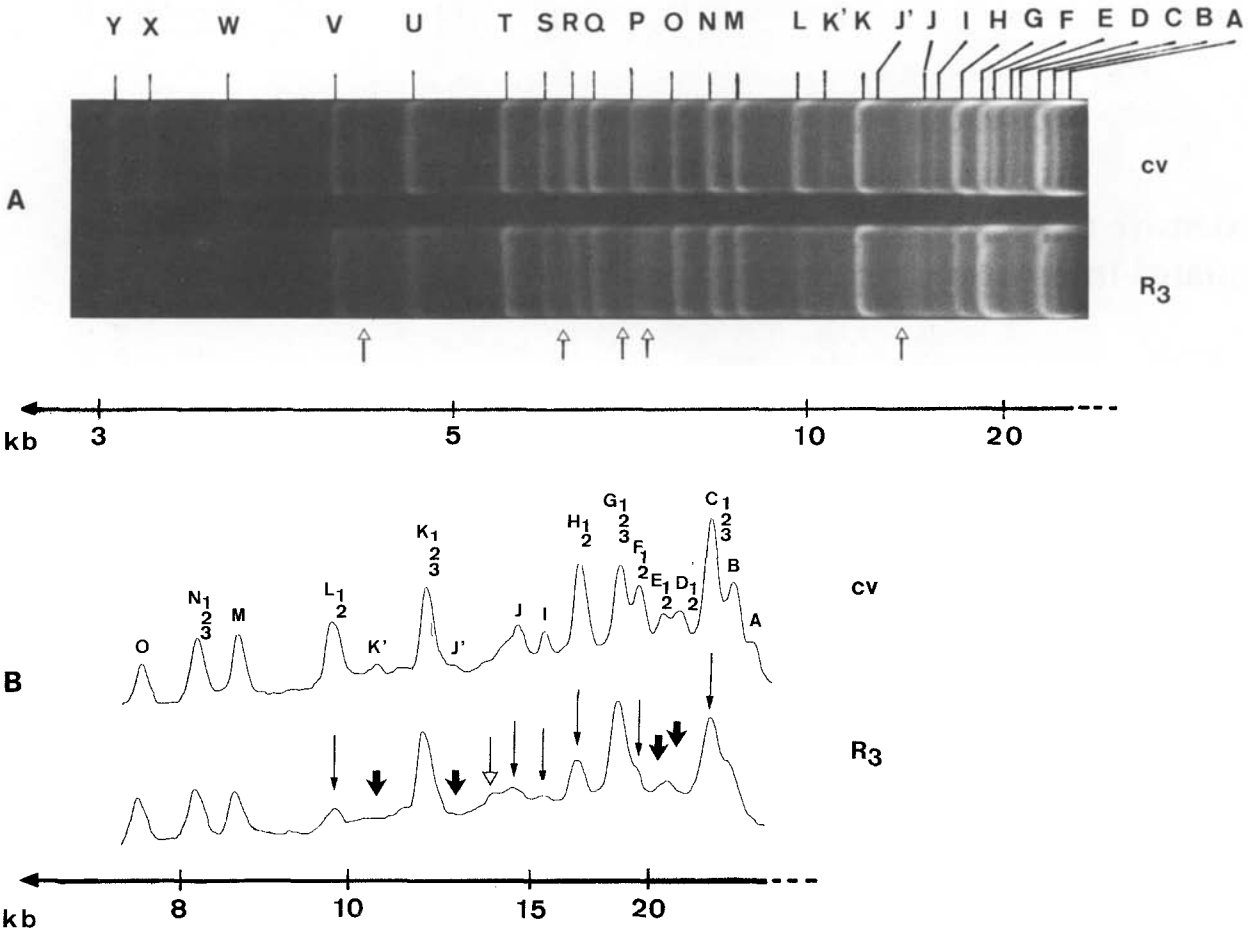

Fig. 1. Electrophoretic characterization of mtDNA rearrangements in wheat callus cultures. mtDNA isolated from parental line (cv) and nonembryogenic calli having undergone 3 subcultures (*R3*) on fresh culture medium was restricted by SalI and electrophoresed in a 0.8% agarose *vertical* gel slab. **A** Electrophoretic patterns from restriction *band A* to restriction *band Y*. **B** Microdensitometer tracings of the region of the electrophoretic patterns located between restriction *band A* and restriction *band 0* ➔ mtDNA fragments not detected in callus cultures; ⟶ decrease in the relative stoichiometry of a given mtDNA restriction band in callus cultures; ⟶▷ cpDNA contamination detected as a distinct peak or shoulder

recombinant cosmid library of wheat mtDNA are consistent with the occurrence of ten sets of different repeated sequences within the mitochondrial genome (Quétier et al. 1985a) which may induce intramolecular recombination and generate a heterologous population of recombining molecules. A knowledge of the ordering of the different SalI fragments contained in each cosmid and the overlapping between these cosmids have allowed Quétier et al. (1985b) to draw a putative master molecule containing all the unique sequences and at least one of each restriction fragment encompassing a given repeated sequence. It should be stressed that a SalI restriction fragment which does not share homology with any other SalI fragment is considered as unique and, as such, is present only once within the master molecule; nevertheless, it can be found in several different subgenomic molecules.

We have used several SalI cloned fragments belonging to a well-defined region of the wheat mtDNA as labelled probes to compare the structural organization of mtDNA from nonembryogenic callus cultures of wheat (*T. aestivum* cv "Aquila") initiated from immature embryos and from the corresponding control parental cultivar. It is clear that in vitro cell culture induces the complete loss of a fraction of the mitochondrial genome. Moreover, it seems likely that this eliminated fraction is located in a subgenomic molecule.

Material and methods

Somatic tissue culture. Glasshouse grown plants of *Triticum aestivum* cv "Aquila" were used as starting material. The immature seeds were harvested 12 to 16 days after anthesis and surface-sterilized in 4% Na-hypochlorite. The immature embryos were excised under a dissecting microscope and placed 10 per 9-cm-petri dish on agar medium with the scutellum uppermost and the meristem embedded in the medium. The basic medium contained the Murashige and Skoog (1962) inorganic salts and

vitamins supplemented with sucrose (20 g/l), 2,4-D (2 mg/l) and agarose (6 g/l). The pH was adjusted to 5.8 before autoclaving. Cultures were maintained at 27° ± 1 °C without illumination. Calli obtained after 7–8 weeks (referred to as R0) were subcultured on the same medium and were referred to as R1, R2 ... Rx. The ability of these calli to regenerate was checked by transfer onto a regeneration medium (MS medium devoid of 2,4-D) but, to date, such attempts to regenerate plants from these calli have been unsuccessful.

Mitochondrial DNA isolation. Plant mtDNA was prepared as previously described (Rode et al. 1985). Callus mtDNA was prepared essentially as described for the whole plant except that the homogenization step was 2 x 3 sec at the low speed of the Waring blender homogenizer or 5 min using mortar and pestle. The yield of mtDNA per fresh weight was about 10-fold higher for callus cultures than for whole plants.

Mitochondrial DNA cloning. Wheat (*T. aestivum* cv "Capitole") mtDNA cosmid cloning has been described elsewhere (Falconet et al. 1985).

Electrophoresis, Southern blotting and DNA-DNA molecular hybridizations. One to 3 μg of mtDNA (calli and control parental line) were digested to completion by SalI and HindIII in the presence of 4 mM spermidine and in a total volume of 20–30 μl. The restriction fragments were separated by electrophoresis on 0.8% agarose vertical slab gels in TEA buffer. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml) and photographed under UV light. The negatives of the photographs were scanned on a double-beam microdensitometer (Joyce-Loebl). Gels were treated as described by Southern (1975) and DNA was transferred onto nitrocellulose filters (Schleicher and Schuell, BA 85) by the sandwich method (Ketner and Kelly 1976). Cosmids were prepared by the boiling method (Holmes and Quigley 1981) followed by CsCl-ethidium bromide ultracentrifugation and labelled by nick-translation (Rigby et al. 1977).

For DNA-DNA molecular hybridizations, nitrocellulose filters were preincubated in a 2 x SSC – 1 x Denhardt solution and allowed to hybridize at 42 °C for 14–16 h in a 2 x SSC – 45% formamide (v/v) – 100 μg/ml carrier DNA mixture containing the nick-translated mtDNA probe. After hybridization, filters were washed in 2 x SSC (6 x 15 min at room temperature) then in 0.01 x SSC (2 x 15 min at room temperature) and dried. Autoradiographs were generally made at –80 °C for 1 to 3 days, using X-Omat AR5 Kodak films and an intensifying screen. Filters were occasionally dehybridized by soaking in boiling distilled water for 8–10 min and subsequently checked for efficiency of dehybridization.

Results

mtDNA SalI cleavage patterns

Samples of mtDNA prepared from parental cultivar (cv) and callus cultures at their third subculture (R3) were digested by SalI and electrophoresed (Fig. 1A). SalI restricted wheat chloroplastic DNA was also electrophoresed in order to detect cpDNA contamination in mtDNA preparations (not shown here). The SalI restriction pattern of *T. aestivum* cv "Capitole" displays 36 visible bands which have been lettered A to Z and then AA to AG with, in addition, 3 faint bands lettered J′, K′ and U′ (Quétier et al. 1985b). Whereas 23 bands correspond only to one SalI fragment each (i.e. A, B, I ...), 13 bands are composed each of several comigrating SalI fragments (i.e. C, D ..., lettered C1, C2, C3 and D1, D2). This nomenclature has been adopted for *T. aestivum* cv "Aquila" as the SalI restriction patterns of both cultivars are qualitatively and quantitatively strictly identical (not shown here). The region of the gel corresponding to restriction bands A to O was scanned in order to obtain a better resolution (Fig. 1B). At first sight, three kinds of differences may be detected (from band A to band Y) between the control parental line and callus mtDNA: first, the disappearance of two single bands (composed of only one restriction fragment, i.e., K′ and J′) and two multiple bands (composed of more than one restriction fragment, i.e., D and E); second, the appearance of a restriction band at about 23 kb (length intermediate between D and E bands, about 25 and 21 kb respectively) and, third, a decrease of some single bands (i.e., I, J and W) and of some multiple bands (i.e., C. F, H and L) in their relative stoichiometry. No detectable differences have been found for bands Z to AG (not shown here).

Taking into account the apparent complexity of the rearrangements occurring in wheat callus culture mtDNA we have chosen to focus our attention on the disappearance of the restriction fragments K′ and J′ – whose structural and coding properties have already been studied elsewhere (Falconet et al. 1985) – keeping in mind that these SalI fragments carry the same repeated sequence.

Molecular hybridizations with SalI restricted mtDNA

As mentioned above, Quétier et al. (1985a) have shown that the wheat mitochondrial genome contains ten sets of repeated sequences. Indeed, molecular hybridization of SalI restricted mtDNA with each of the SalI fragments of the mtDNA library identifies either only the corresponding SalI mtDNA fragment (unique sequence) or four fragments, including the SalI fragment used as a probe (except if a SalI site is located within the repeated sequence: in this case, only two bands light up). In the latter case, the observed hybridization pattern indicates the occurrence of an intramolecular recombination phenomenon at the level of the same repeated sequence. As shown in Fig. 2, the SalI mtDNA fragment K′ encloses repeated sequence nr. 5. This fragment, as well as the fragment K1, have been shown to arise from an intramolecular recombination phenomenon between

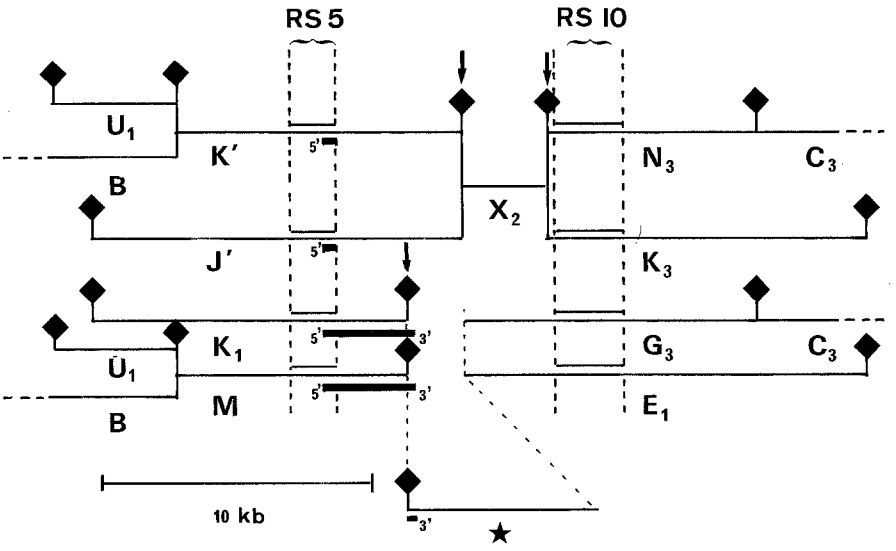

Fig. 2. Arrangement of the cloned mtDNA SalI fragments encompassing the repeated sequence nr. 5 and the repeated sequence nr. 10. The fragments *K'*, *J'*, *K1* and *M*, on the one hand, and the fragments *N3*, *K3*, *G3* and *E1*, on the other hand, have been aligned on the scheme with respect to repeated sequence nr. 5 and repeated sequence nr. 10 respectively. Under these conditions, G3 and E1 could not be entirely represented (⟨). However, this omitted part of G3 and E1 is shown on the lower part of the scheme (★). *RS5* and *RS10:* repeated sequences nr. 5 and nr. 10, where intramolecular recombination can occur; ♦ SalI site (Falconet et al. 1984); —— Location of the 26S ribosomal gene and position of its 5' and 3' ends (Falconet et al. 1985); ↓ Location of the SalI sites where multiple possibilities of SalI fragment arrangements can occur (K'–X2 and J'–X2; X2–N3 and X2–K3; K1–G3 and K1–E1; M–G3 and M–E1). These different possibilities have been found in different cosmid clones. The SalI fragments flanking K' and M (U1 and B), N3 and G3 (C3) have been placed on this scheme. Their sequential order has also been obtained from cosmid cloning data

the fragments M and J' (or vice versa) which both contain the repeated sequence nr. 5 (Falconet et al. 1985). The fragments N3 and K3 – separated from fragment K' only by fragment X2 (cf. Fig. 2) – also contain a repeated sequence (nr. 10). Thus, a simple explanation for the disappearance of fragments K' and J' was the possibility of the complete loss of the total length of DNA located between repeated sequences nr. 5 and nr. 10 (i.e. the fragment X2 and the section of fragments N3 (or K3) and K' (or J') internal to these repeated sequences). To check this hypothesis, SalI restricted mtDNA from callus culture R3 and control parental line were hybridized with the labelled SalI mtDNA fragments K', X2 and N3 and both flanking fragments C3 and B (cf. Fig. 2). As expected from restriction patterns, the fragments K' and J' are absent in callus culture mtDNA (Fig. 3) even after a prolonged exposure (not shown here). It must be underlined that, among the four fragments K', J', K1 and M related to one another by intramolecular recombination (and lit up by K' in control parental line), the two fragments K' and J', absent in callus cultures, are those which enclose only the 5' part of the 26S rRNA gene, located in the repeated sequence (cf., Fig. 2).

Hybridization with the labelled SalI mtDNA probe X2 (Fig. 3), which does not share homology with any other SalI fragment, clearly shows that this fragment is also missing in callus culture mtDNA.

Hybridization with the labelled mtDNA probe N3 gives results similar to those obtained with K'. Indeed, fragment N3 (cf. Fig. 2) contains a repeated sequence (nr. 10) which enables an intramolecular recombination between N3 and G3, in turn generating E1 and K3 (unpublished results). The hybridization pattern of parental line restricted mtDNA (Fig. 3) agrees with this finding whereas that of the callus culture shows the absence of both fragment N3 and of recombining fragments E1 and K3. However, two faint bands located between G3 and K3 and between K3 and N3 respectively are visualized on the parental line mtDNA hybridization pattern. Their origin is still unclear although we have recently found that the SalI fragment located between G3 and K3 could be detected when probing with a 2.1 kb AvaI-AvaI mtDNA fragment internal to repeated sequence nr. 10 (not shown here). Thus, of the four major mtDNA fragments enclosing repeated sequence nr. 10, only fragment G3 persists in callus cultures. It should be noted that G3 encloses the 3' end of the 26S rRNA gene (cf., Fig. 2). Under these conditions, at least one entire 26S rRNA gene persists in callus culture mtDNA (the K1–G3 and M–G3 arrangements, effectively found in cosmid cloning).

Since N3 and K' are respectively flanked by C3 and either U1 or B (Fig. 2), we hybridized SalI restricted callus and control mtDNA with C3 and B in order to determine whether or not these fragments were also

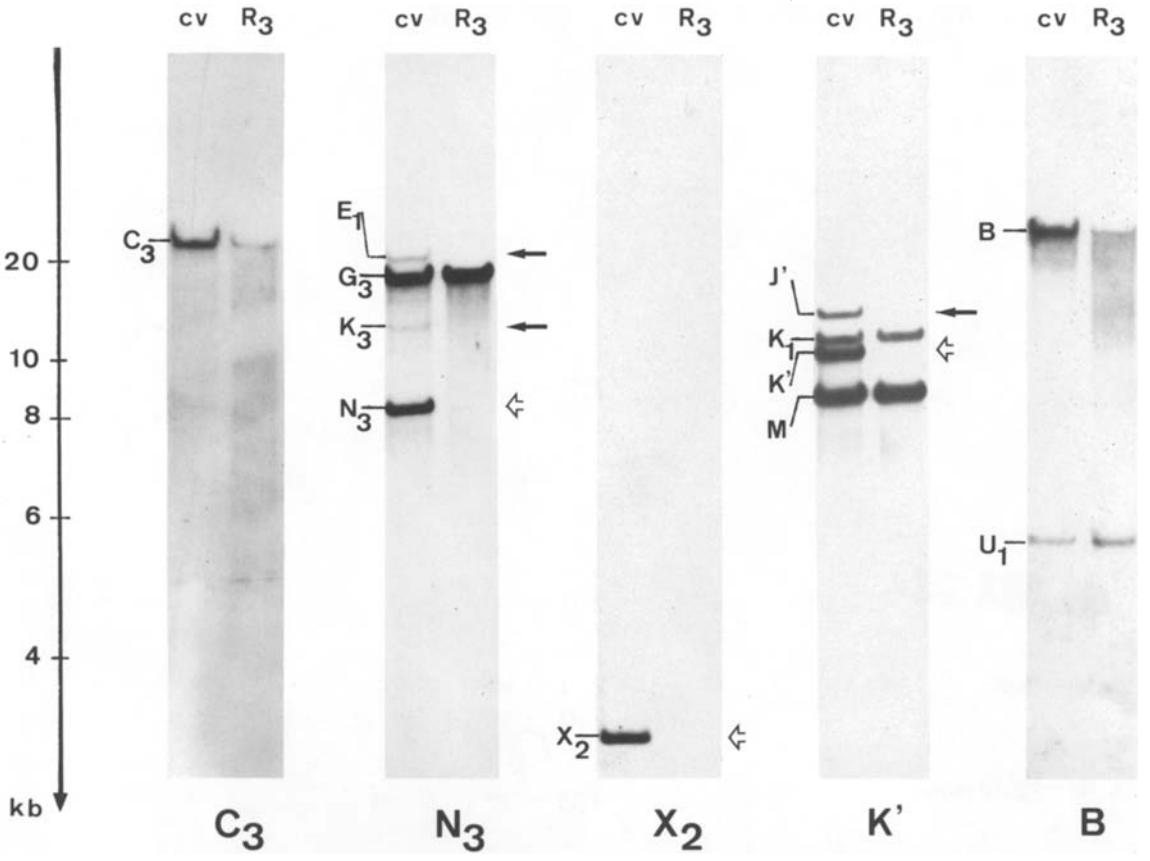

Fig. 3. DNA-DNA hybridizations between SalI restricted mtDNA isolated from parental line (*cv*) and callus cultures at their third subculture (*R3*) with labelled SalI mtDNA probes C3, N3, X2, K' and B. ⇨ the mtDNA fragment corresponding to the probe cannot be detected in callus cultures; → a mtDNA fragment arising by a reciprocal recombination process involving the mtDNA fragment corresponding to the probe cannot be detected in callus cultures

missing in callus cultures (Fig. 3). A hybridization band corresponding to the probe itself is effectively detected in each case. However, the intensity of both C3 and B is considerably lower than that found in the control parental line. In addition, hybridization with the labelled probe B also lights up, as expected, the restriction band U in both the control parental line and callus cultures (Fig. 3). This is not surprising as the SalI site separating U1 and B from either K′ or M (cf., Fig. 2) is located in repeated sequence nr. 6 (unpublished results). Under these conditions, fragment B, used as a labelled probe, can only light up fragment U1 in addition to itself.

These results deal with a callus culture in its third subculture. Identical results on hybridization of SalI restricted mtDNA with the labelled fragments K′, X2 and N3 have been obtained with callus cultures in their first and seventh subculture (not shown here). This fact suggests that first, these mtDNA variations occur during the early process of callogenesis and, second, these variations are conserved throughout successive subcultures. Work is currently underway to identify the time-course of the disappearance of fragments such as K′, X2 and N3 from initiation of callus cultures to the R0 stage.

Molecular hybridizations with SalI + HindIII restricted mtDNA

The HindIII physical map of both sets of SalI fragments containing repeated sequences nr. 5 and nr. 10 as well as their arrangement with respect to these repeated sequences (Falconet et al. 1985) can validate the results mentioned above. For this purpose, SalI + HindIII double digests of callus culture R3 and control parental line mtDNA have been hybridized with the labelled fragments N3 and K′ (Fig. 4). The results obtained with the labelled probe K′ demonstrate that all the expected HindIII and HindIII–SalI fragments deduced from the restriction map are effectively visualized in the parental line whereas the fragments belonging only to K′ and J′ (the 3.9 kb HindIII and 2.1 kb HindIII–SalI fragments) are missing in callus cultures.

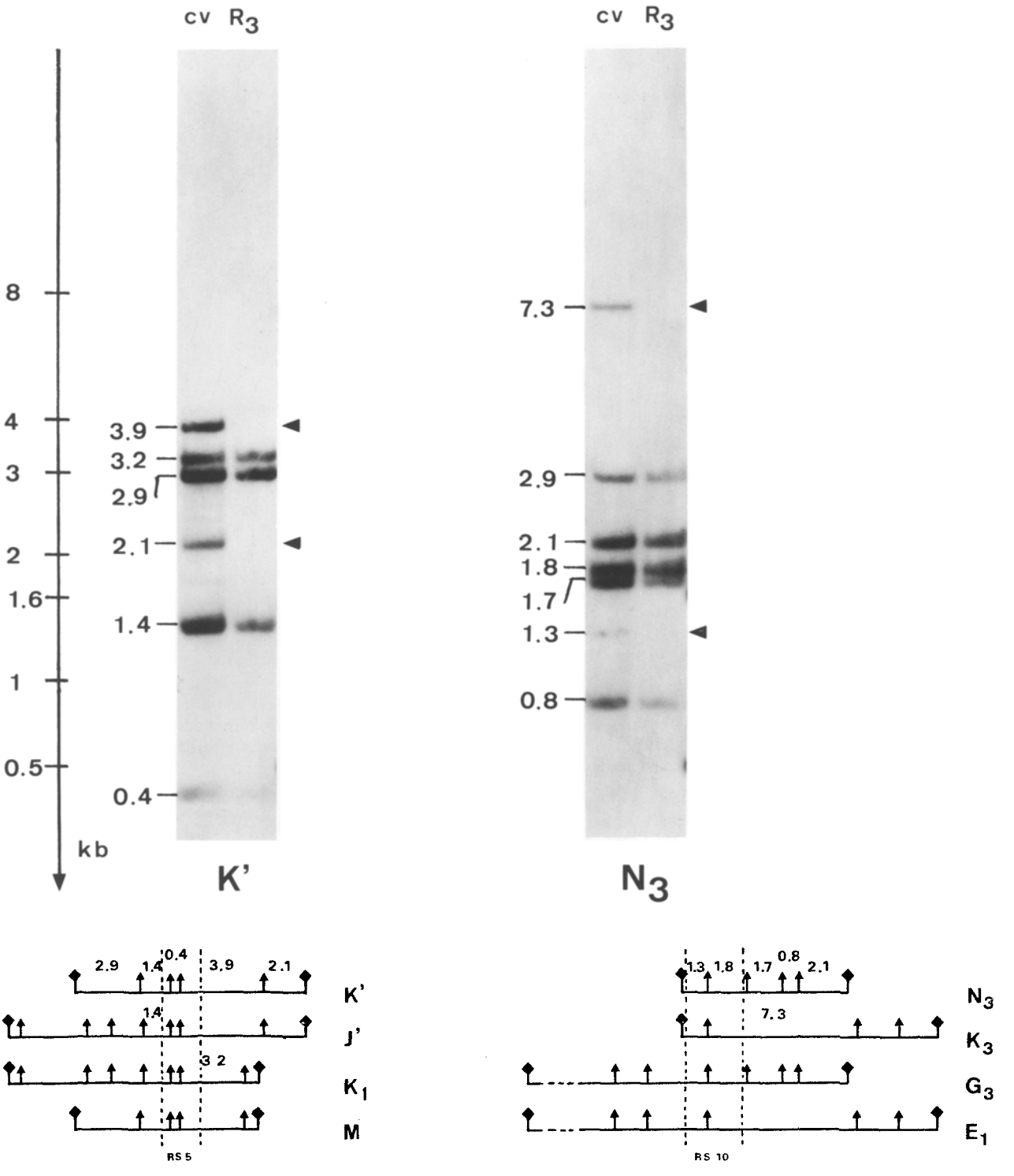

Fig. 4. DNA-DNA hybridizations between SalI + HindIII double digests of mtDNA isolated from parental line (*cv*) and callus cultures at their third subculture (*R3*) with labelled SalI mtDNA probes K' and N3. The lengths of the different hybridization bands are in kb. Under each hybridization pattern is presented the HindIII restriction map of the SalI mtDNA fragments encompassing either repeated sequence nr. 5 or repeated sequence nr. 10. The lengths (kb) of the different HindIII and HindIII-SalI fragments are indicated above each fragment

The same results are obtained when fragment N3 is used as a probe: the 7.3 kb HindIII fragment which belongs only to K3 and E1 is missing as is the 1.3 kb HindIII–SalI fragment belonging only to N3 and K3. Only fragments coming from G3 and comprising a sequence common to N3 are still present in callus cultures.

Discussion

Molecular hybridizations using selected fragments of a wheat SalI mtDNA library have allowed us to detect marked variations in the organization of the wheat mitochondrial genome of nonembryogenic calli initiated from immature embryos. In this paper we have described variations concerning a region of the mitochondrial genome enclosing a gene whose intramolecular localization has already been determined: the 26S rRNA gene (Falconet et al. 1985). As reported by these authors, the entire 26S rRNA gene is flanked by four different sequences, suggesting that these fragments are related by homologous recombination within a common repeated sequence. It ensues that this gene is present in multiple copies in wheat mtDNA. Arrangements enclosing the entire gene, such as K1–G3, K1–E1, M–G3 and M–E1 (cf. Fig. 2), have effectively been found. On the other hand, fragments such as K′ and J′ contain only the 5′ end of the 26S gene. Our results have shown that among the eight fragments encompassing either repeated sequence nr. 5 or repeated sequence nr. 10, only K1, M and G3 were still present in callus culture mtDNA. It is noted that both arrangements K1–G3 and M–G3 allow the entire 26S gene to still be present in two copies which would then be sufficient to permit enough 26S rRNA to be transcribed. However, the question arises as to whether all the 26S gene copies found in the differentiated plant are expressed or not, as recombination has been shown to alter gene expression in other systems (Zieg et al. 1979; Seidman et al. 1979).

When compared to the corresponding plant, wheat callus cultures may be considered as mitochondrial variants whose genome complexity has been lowered due to the disappearance of a fraction of this genome. Mitochondrial DNA in higher plants is thought to be composed of differently sized circular and linear molecules (Leaver and Gray 1982; Quétier et al. 1985a) if the latter do not represent a category of molecules generated by in vitro treatments. The disappearance of a part of the genome in callus cultures may be a priori explained by either a deletion of defined parts of some of these molecules or a selective loss of one (or several) of these molecules. If part of a subgenomic molecule was lost, a new fragment should appear having the newly connected borders of the deletion. Under these conditions, such a new fragment could be visualized when probing either SalI + HindIII (Fig. 4) or SalI (Fig. 3) Southern blots with each of the two SalI fragments where the excision would have taken place. Such a hypothetical band has never been found in this system, thus favouring the hypothesis of a complete loss of one (or several) mtDNA subgenomic molecule(s) in response to in vitro culture.

An intriguing feature lies in the fact that the two SalI fragments B and C3, which have been shown to flank K′ and N3 respectively, are still present – although in minute amounts – in callus culture mtDNA. However, this finding agrees with the fact that fragments such as B and C3 might be present in the parental cultivar within two or more mtDNA subgenomic molecules. According to this hypothesis, the fraction of the mtDNA genome including B and C3 and located within a subgenomic molecule encompassing K′, X2 and N3 would have disappeared whereas the same arrangement, located within another subgenomic molecule still present in callus cultures, would be responsible for the weak hybridization band observed. This hypothesis is supported by the fact that restriction fragment B can be found in the arrangements B–K′ and B–M (Fig. 2) due to the presence of a repeated sequence (nr. 6) which overlaps these arrangements.

If some of the forms of callus culture mtDNA subgenomic molecules are effectively eliminated from the mitochondrial genome, how may one explain this disappearance? A possible explanation would be that in vitro culture is able to induce some differential replication rates among the various mtDNA subgenomic molecules due to selection pressure. Some molecular populations would not be affected by an in vitro culture process and would then replicate like mtDNA of the control parental line whereas other populations would slow down their replication rate to a point where they would be diluted and thus progressively eliminated. Unfortunately, the molecular basis of higher plant mtDNA replication is poorly understood: Two reports, however, deal with the proportion of circular molecules which is higher in mtDNA extracted from liquid cultured cells than in mtDNA extracted from plants (Dale et al. 1981) and with the relative concentration of a plasmid sequence versus a sequence carried by large molecules which is different between maize leaves and anthers (Abbott et al. 1985).

As far as wheat mtDNA is concerned, it seems that in vitro culture of somatic cells leads to reduced genomic complexity. This feature seems to be a characteristic of somatic cell cultures since all the anther-derived dihaploid lines of wheat (Rode et al. 1985) and triticale (Charmet et al. 1985) so far studied have

exactly the same mitochondrial genome as the corresponding parental line.

Finally, does the disappearance of a fraction of the mitochondrial genome only illustrate selfish or parasitic DNA or does it relate, at least partly, to the inability of this callus culture to regenerate? Sets of embryogenic and nonembryogenic calli of wheat are being initiated to check whether the same deletion will be detected. On the other hand, the only gene which is known until now to be located in the stretch of DNA involved is the one coding for the 26S rRNA; two copies carried by the K1–G3 and M–G3 arrangements still persist in the nonembryogenic callus culture. Attempts to detect open reading frames in the deleted DNA are underway, as well as possible mRNA transcripts using RNA blots.

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